Research Article

Equine Herpes Virus 4 (EHV4) Investigation in Aborted Egyptian Mares; Molecular Detection, Isolation, and Phylogeny for Viral Glycoprotein B

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Abstract | An abortion outbreak was reported in horse studs, Egypt, in 2019. Investigating the role of EHV-4 in this abortion outbreak in mares using various molecular detection techniques were done using the tentative diagnosis by rt-PCR. The presence of 16 liver and spleen samples out of 20 (80%) from aborted fetuses were positive for EHV-4, but all were negative for EHV-1. Virus isolation trial for EHV-4 were done, eleven samples out of 20 (55%) on the CAM of ECE were positive. The virus glycoprotein B (gB) fragment (580pb) was amplified in selected isolates using the nested PCR (n-PCR), sanger sequencing of gB from three isolates with phylogenetic analysis reveals the full identity between the Egyptian isolates from the outbreaks and other EHV-4 strains available in databases proving wide distance with other EHV-8 and EHV-1. The study recommends rt-PCR as a screening test for the tentative diagnosis of EHV-4 in epidemiological studies and n-PCR as a sensitive differential test for the virus detection. The virological studies and molecular assays confirm the involvement of EHV-4 in equine abortion, suggests the possibility that latent virus reactivation in mares could result in abortion due to certain factors, including stress more epidemiological investigation. Whole-genome sequencing is required to address any genetic recombination in EHV-4 associated with abortion cases.

Keywords | EHV-4, Equine, rt-PCR, n-PCR, Sequencing, Phylogenetic analysis, Outbreak

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INTRODUCTION

Equine herpesviruses (EHVs) have a great economic impact on the horses' industry. They are widely spread globally and are responsible for various diseases in the horse populations (Slater et al., 2006; Telford et al., 1998). Nine herpesviruses have been identified in equids; six belong to the subfamily *Alphaherpesvirinae* Equine herpesvirus 1 (EHV-1, equine abortion virus), EHV-3 (equine coital exanthema virus), EHV-4 (equine rhino pneumonitis

virus), EHV-6 (AHV-1, asinine herpesvirus 1), EHV-8 (AHV3, asinine herpesvirus 3) and EHV-9 (gazelle herpes virus), and three; Equine herpesvirus-2 (EHV-2), Equine herpesvirus-5 (EHV-5) and Equid herpesvirus-7 (EHV-7 or asinine herpesvirus 2) are all members of the *Gammaherpesvirinae* (Davison et al., 2009).

EHV-1 and EHV-4 belong to the same genus *Varicellovirus*, regarded as the most important equine herpesviruses. They are genetically and antigenically linked, with cross-reactivity. Antigenic variants, on the other hand, were discovered as early as 1959, and molecular investigations and specific gene sequencing recently revealed genetic differences, and they are two distinct viruses (Harless and Pusterla, 2006; Sabine et al., 1981).

Both EHV-1and EHV-4 viruses have a linear, double-stranded DNA molecule of 150 kbp and 146 kbp in length, respectively; the viral genome structure of the two viruses is composed of a long unique region (U.L.) flanked by a short inverted repeat (TRL/IRL) linked to a unique short area (U.S.) flanked by a substantially inverted repeat (TRS/IRS) (Telford et al., 1992; Telford et al., 1998). EHV-1 genome encodes 80 open reading frames (ORFs), while EHV-4 encodes 79 ORFs. Both EHV-1 and EHV-4 are genetically related viruses with a range of 54.9–96.4% amino acid sequences prediction of their genes (Telford et al., 1998).

EHV1 and EHV-4 are the main viruses responsible for equine viral respiratory diseases with equine influenza virus (EIV) (Pusterla et al., 2011). EHV-1 infection is manifested with respiratory signs, abortion, neonatal foal death, Myelencephalopathy (Slater et al., 2006; Zarski et al., 2021).

EHV-4 causes respiratory diseases in foals two years old or younger and rarely causes abortion (Matsumura et al., 1992; Telford et al., 1998). Still, the EHV-4 becoming latent in the trigeminal ganglion of infected animals is the major outcome and could be reactivated periodically, inducing infection (Borchers et al., 1999; Browning et al., 1988).

A key method for transmitting infection among susceptible animals is asymptomatic carriers' intermittent recrudescent viral shedding (Patel and Heldens, 2005). Many serological surveys indicated heavy exposure to the virus where more than 80% seroprevalence among horses, donkeys, and mules was detected in different geographical areas (Gilkerson et al., 1999; Mekonnen et al., 2017; Yildirim et al., 2015).

EHV-4 diagnosis of infection and shedding is usually based on molecular detection by polymerase chain reaction (PCR) or quantitative PCR (qPCR) of nasal swabs/washes (Pusterla et al., 2017). Several PCRs using unique primers were intended to identify EHV-1 and EHV-4 in clinical samples, tissues, or cell lines that had been injected with the virus (Dynon et al., 2001; Lawrence et al., 1994; O'Keefe et al., 1994; Wagner et al., 1992). The multiplex PCR and rt-PCR can simultaneously detect and discriminate EHV-1 and EHV-4 from each other (Ataseven et al., 2009; Diallo et al., 2007; Ghoniem et al., 2018).

Serological assays were also developed and validated, such as specific ELISA (Crabb et al., 1995; Hartley et al., 2005) and peptide ELISA (Lang et al., 2013), virus neutralization test (VNT) (OIE, 2018); also virus isolation on primary cell culture and are considered the gold standard assay for laboratory diagnosis and facilitate the comparative and molecular analyses (Lunn et al., 2009; Pavulraj et al., 2021), gene sequencing and whole-genome sequencing are becoming more available and used to identify genetic variations in isolated viruses by phylogenetic analysis (Izume et al., 2017; Telford et al., 1998; Vaz et al., 2016).

In Egyptian economics, horses play a vital role; horses represent about 40% of the estimated 1.5 million equids, according to the Egyptian Ministry of Agriculture, Animal Wealth Development Sector, 2010. EHV-1 circulation has been documented on numerous occasions after it was isolated on two distinct occasions from aborted fetal organs on Chorioallantoic membrane (CAM) of embryonated chicken Eggs (ECE) and cell lines and confirmed by PCR and ELISA (Ahmed et al., 2015; Amer et al., 2011; Warda et al., 2003), few reports are available regarding the EHV-4 and its role in abortion (Afify et al., 2017; Ahmed et al., 2015; Azab et al., 2019).

Because the prevalence of abortion induced via EHV-4 in the field is unknown in Egypt, we performed this study to evaluate the involvement of EHV-4 in the horse abortion outbreak in Egypt, virus isolation and molecular characterization of virus, sequencing and phylogenetic analysis for comparing the sequence of gB isolated from abortion cases and other reported from respiratory one.

MATERIALS AND METHODS

Ethical approval

The study was carried out following the Animal's applicable guidelines and regulations in Egypt. No equids were experimentally used or euthanized for this study, and samples were collected post-mortem with prior owners' consent. All the diagnostic methods and laboratory work were followed the viral isolation, biosafety, and quality standards of AHRI, ARC, Dokki, and Giza, Egypt.

Samples and study area

Tissue samples (n = 20) from aborted fetuses (liver and

spleen) and mares were immediately collected aseptically after abortion outbreak notification from horses' studs in Cairo province, Egypt, during 2019. Samples were transported chilled in proper containers directly to the departments of Virology, biotechnology AHRI, Dokki, and Giza Egypt. It was processed and prepared for investigation of EHV-4 following (OIE) guidelines for EHVs Isolation and identification (OIE, 2018).

Viral DNA extraction

For molecular purposes in this study, Viral genomic DNA was extracted from tissue samples, and isolates showed CPE using the Gene Jet genomic DNA extraction kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions.

Molecular detection of EHV-4 by qPCR

Samples from aborted fetuses were initially screened for both EHV-4 in with EHV- 1 using Power CheckTM Equine Herpesvirus type 1 and 4 probe-based qPCR Kit (Kogenbiotech, Seoul, Korea) using total reaction volume 20 µl containing specific primer and probe 4 µl,2x PCR master mix and 5 µl of extracted DNA template or positive control supplied with Kit and nuclease-free water for a negative one, the EHV-1 detected on FAM channel while EHV-4 ON VIC or HEX, all steps were performed according to the instructions included with the kit.

Virus isolation

Virus isolation was done by inoculating specific pathogen-free embryonated chicken Eggs (SPF-ECE) 9-11 days old on CAM (Hassanien et al., 2002). Those that died within 24 hours of being injected were discarded. Death after 24 hours post-inoculation (P.I.) was classified as non-specific.

EHV-4 isolates confirmation by nested PCR

The viral DNA from positive isolates was identified using a nested-PCR (n-PCR) developed by (Borchers and Slater, 1993) and approved by the OIE. The specific primers amplify 580 pb of EHV-4 glycoprotein B (gB) gene target that allows differentiating EHV-4 from EHV-1 were synthesized in (Thermo scientific, USA), the PCR test performed in bio rad thermocycler using a 20 µl as a total reaction volume; 3.5µl of DNA template added to 12.5µl PCR mixture 1x PCR buffer (Amplitaq gold 360® Mix, 2µl from each oligonucleotide primer (20Pmol) in both stages of amplification as recommended by the manufacturer, the primer sequences and amplification conditions are shown in the Table 1. After electrophoresis in % agarose with 10 g/ml ethidium bromide, the PCR products were visualized under ultraviolet light, and the amplification was confirmed.

EHV-4 gB DNA sequencing and phylogenetic analysis

Three viral isolates were selected for genotyping of gB, QIAquick® Gel Extraction Kit (QIAGEN, Germany) was used to purify the PCR products from the second n-PCR stage and then sequenced in both directions with the identical primers and conditions in the AHRI following manufacturer's instructions for BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, USA). ChromasPro software was used to remove low-quality portions of the gB gene sequence (ChromasPro 1.7, Technelysium Pty Ltd., Tewantin, Australia). The gB sequences were fetched in the National center for biotechnology information (NCBI) using a Basic local alignment tool for nucleotide (BLASTN), and highly similar sequences for EHV-4 and EHV-8 EHV-1 were selected and downloaded in fatsa format. Multiple sequence alignments (MSA) were performed using the UGEN software and MUSCLE algorism to investigate the evolutionary relationship between the sequenced gene from the current EHV-4 outbreak and other isolates and strains available in the GenBank database (Okonechnikov et al., 2012).

IQ-TREE was used to create the phylogenetic tree (Nguyen et al., 2014), best model finder, and 1000 bootstrap repetitions were used to determine the most accurate model while using the maximum likelihood method. The tree was rescaled to indicate the number of substitutions per site by using branch length as a measure. Display, manipulation, and annotation of the phylogenetic tree were done with the Interactive tree of life (iTOL) (Letunic and Bork, 2021).

Table 1: The EHV-4 oligonucleotide sequences and cycling conditions used in the n-PCR.

n-PCR Stages	EHV-4 primers	Sequence	Cycling conditions (For both steps)		
First stage amplification	BS-4 –P1	5'-TCTATTGAGTTTGCTATGCT 3'	1 cycle	Initial denaturation	94°C /4 min
	BS-4 –P2	5'-TCCTGGTTGTTATTGGGTAT 3'	40 Cycle	Denaturation	94°C/ 30 S
				Annealing	60°C for 30 S,
				Extension	72 °C/90 S.
Second stage amplification	BS-4 –P3	5'-TGTTTCCGCCACTCTTGACG 3'		Final extension	72°Cfor10 minutes
	BS-4 –P4	5'-ACTGCCTCTCCCACCTTACC 3'			

RESULTS AND DISCUSSION

Outbreaks presentation and molecular detection

The rapid molecular screening with probe-based rt-PCR for the 20 samples from aborted fetuses revealed the presence of EHV-4 in 16 samples (80%), and all samples were negative for EHV-1 as in Figure 1.

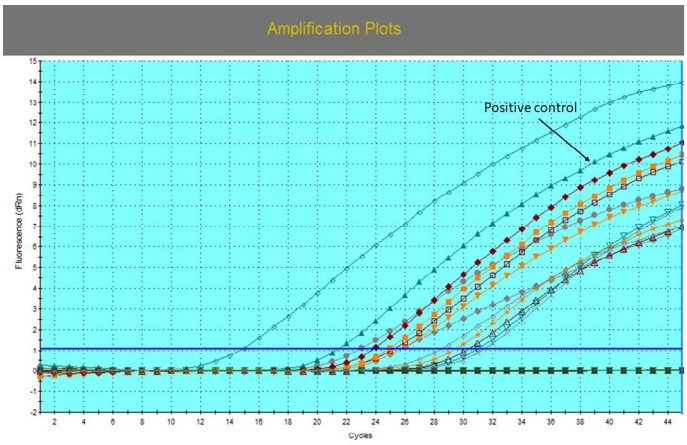

Figure 1: The amplification plot for EHV-4 qPCR with different threshold cycles (Ct) values for 16 positive samples. EVHV-4 stander was used as a positive control with Ct 18.

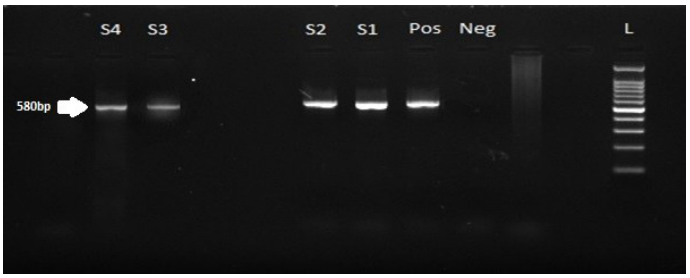

Figure 2: Nested PCR gel electrophoretic pattern of the EHV-4 gB gene, the specific band amplified at the expected size 580bp where: lanes S1, S2, S3, S4 for samples, L 100bp for Ladder, Pos for positive control, and Neg for the negative specimen.

Virus isolation

The trial for Isolation of EHV-4 from infected animals on CAM of ECE was successful in eleven samples with high cycle threshold (C.T.) value in rt-PCR, all positive samples the cytopathic effect (CPE) characterized by thickening or diffuse opacity of CAM with the presence of necrotic pock lesion within 5-6 days PI, these lesions were increased with the progressive virus passages till 3rd passage due to the increase of the viral load in isolates. The samples with a low C.T. value in rt-PCR are not propagated in ECE as they contain a low viral load.

Identification of the isolates by n-PCR and sequencing

The genomic DNA of the EHV-4 gB gene was successfully detected by n-PCR, where the target 580 pb fragment was amplified in positive isolates Figure 2, the selected three isolates were sequenced, and the high-quality nucleotide sequences of partial gB gene obtained were deposited in GenBank with accession numbers MW039139, MW039140, MW039141.

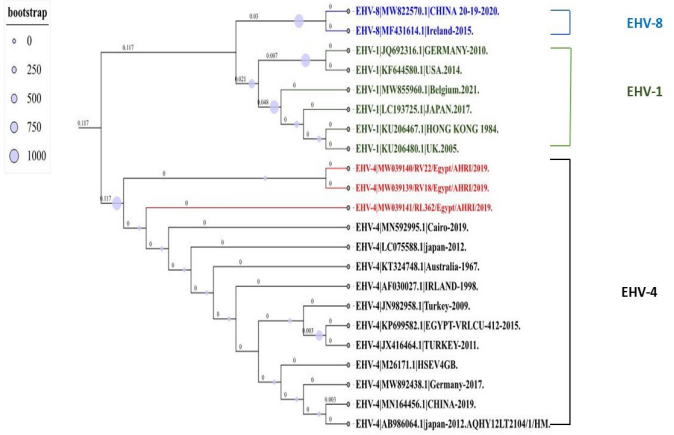

Figure 3: The maximum likelihood Phylogenetic tree is based on 409 pb gB gene fragment of EHV-4 isolated from Egypt using IQ-TREE with best Model Finders. The accession numbers with brief GenBank ID are located in each sequence from EHV-4, EH-8, AND EHV-1, The three Egyptian isolates are highlighted in red, and other clades are assigned different colors, and the numbers above the branch represent its length and the bootstrap values computed from 1000 bootstrap repeats. The tree is rooted in the middle.

Phylogenetic analysis

A phylogenetic tree was constructed the gB sequence of our three EHV-4 isolates with other genome sequences retrieved from the GenBank (Figure 3), MSA and phylogenetic analysis revealed sequence 100% identity between the three EHV-4 field isolates in this study with no differences where they have the same nucleotides and amino acid sequences, using partial and complete genome sequences for EHV-4 gB published in GenBank, our isolates are closely related to the isolates from different geographical location showed 100 % identity with isolates from Japan, Australia, Germany, Turkey, China, Ireland and 99% for 1 isolate from Egypt, all clustered together. The phylogenetic tree of gB clustered the Egyptian three isolates with the other eleven EHV-4 isolates. It proved that all have the same gB except for one nucleotide difference from G to C at position 247 in the isolate of China (MN164456.1) and other single nucleotide polymorphism (SNP) from G to A in isolates from Egypt (KP699582.1) and turkey (JX416464.1) at position 405 of our isolate's sequences (Figure 4), and this considers a point mutation needed to be investigated. The MSA showed a significant difference between EHV-4 isolates and other EHV-8 and EHV-1 isolates where they reveal similarity with 87 %

Table 2: Source modifier tabulates for gB sequence records retrieved from GenBank for alignment, phylogenetic analysis, and tree construction for EHV-4 isolates from an abortion outbreak in Egypt.

EHV	Country (location)	Sample sequence ID	GenBank Accession number	Collection date	Host
EHV-4	Egypt	EHV4/RV18/Egypt/AHRI/2019	MW039139	2019	Horse
	Egypt	EHV4/RV22/Egypt/AHRI/2019	MW039140	2019	Horse
	Egypt	EHV4/RL362/Egypt/AHRI/2019	MW039141	2019	Horse
	Egypt	EHV-4|MN592995.1|Cairo-2019.	MN592995.1	2019	Horse
	Japan	EHV-4 DNA, strain: 12-I-203	LC075588.1	2012	Horse
	Australia	EHV-4 isolate ER39-67	KT324748.1	1967	Horse
	IRLAND	EHV-4 strain NS80567	AF030027.1	1998	Horse
	Turkey	EHV-4| isolate US4-TR2011 gB gene	JN982958.1	2009	Horse
	EGYPT	EHV-4 isolate VRLCU-412-2015 gB gene	KP699582.1	2015	Horse
	TURKEY	EHV-4 isolate TR-EHV4-ED-11 gB	JX416464.1	2011	Horse
	IRLAND	EHV-4 gB HSEV4 GB.	M26171.1	1989	Horse
	Germany	EHV-4 isolate DE17_4	MW892438.1	2017	Horse
	CHINA	EHV-4 envelope glycoprotein B gene	MN164456.1	2018	/Horse
	Japan	EHV-4 gB isolate AQHY12LT2104/1/HM	AB986064.1	2012	Horse
EHV-8	CHINA	EHV-8 isolate SD2020113	MW822570.1	2020	donkey
	Ireland	EHV-8 strain EHV-8/IR/2015/40	MF431614.1	2015	donkey
EHV-1	GERMANY	EHV-1 gB gene	JQ692316.1	2010	Polar bear
	USA	EHV-1 strain T-529 10/84	KF644580.1	2014	Onager
	Belgium	EHV-1|strain BE/21P43_BD5/2021	MW855960.1	2021	Horse
	JAPAN	EHV-1 strain: Ab4p_attB_delta_VP22	LC193725.1	2017	Horse
	HONG_KONG	EHV-1 strain Hong Kong/57/1984	KU206467.1	1984	Horse
	UK	EHV-1 strain Suffolk/123/2005	KU206480.1	2005	Horse

for EHV-8 from Ireland and China and EHV-1 from USA, Germany, UK, Hong Kong, Japan, and Belgium, all sequence details in Table 2 and highlighted nucleotides differences in Figure 5.

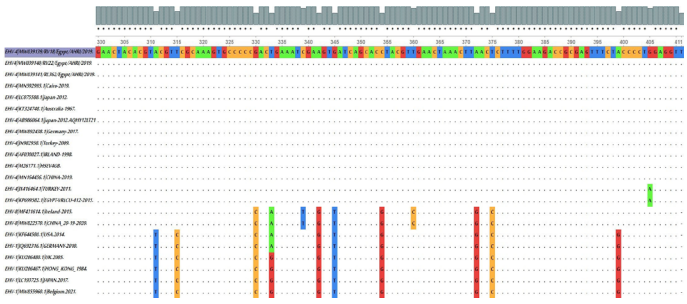

Figure 4: The nucleotide MSA for Partial gB gene from EHV-4 isolates (position 300-411) compared to other EHV4, EHV-8, and EHV1 retrieved from Gene Bank visualized in UGEN software. The dot (.) represents identity, while a single nucleotide highlights the differences.

At the amino acid level, the sequence translation of the gB sequence from the three Egyptian isolates showed 100 % identity with all selected isolates and strains of EHV-4 except single amino acid in Chinese isolate (MN164456.1), where the A in position 83 is replaced with S, in comparison with EHV-1 and EHV-8 there was a mismatch in 8 amino acid positions that explains the 87 % identity Figure 5.

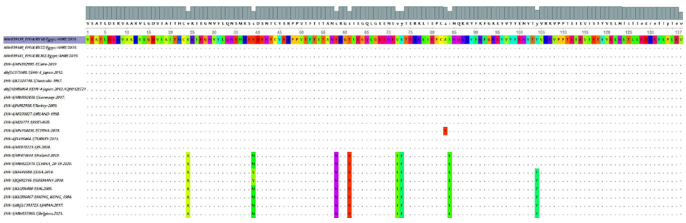

Figure 5: The amino acids MSA for the partial gB gene sequence of the three identified EHV-4 isolates compared to other retrieved EHVs where a single alphabet highlights the differences among amino acids.

This study investigates an outbreak of abortion in equines with detection and Isolation of EHV-4 from aborted fetuses during 2019 in special horse studs at Cairo, Egypt. The direct molecular diagnosis using qPCR confirmed the presence of EHV-4 in 16 samples, and all tested fetuses were negative for EHV-1. The virus causes a large economic loss in horses globally and is mostly presented as a respiratory disease with mild to moderate signs in foals and is apparent in mares and stallions (Pusterla et al., 2011). EHV-4 causes inapparent infections in most cases

even in foals (Smith et al., 2018).

In the current research the virus was detected in the abortion outbreak that confirms the virus contribution in the abortion incidence in mares which is coincide with previous study as isolates were retrieved from liver and spleen samples of aborted foeti (Gerst et al., 2003; Amer et al., 2011; Afify et al., 2017; Al-Shammari et al., 2016; Mohamed et al., 2017) on other hand detection of the virus from nasal swabs is the main known samples which is in agree with EHV-4 pathogenicity as reported (Amer et al., 2011; Gohneim et al., 2018) and confirm the high prevalence of the virus among horse populations (Amer et al., 2011; Gilkerson et al., 1999) , we detected only EHV-4 as a cause of abortion in this molecular investigation where 16 samples were positive by an rt-PCR, however, mixed infection of both EHV-1/-4 has been previously detected (Taktaz-Hafshejani et al., 2015), preliminary assay for detection EHV-1/-4 is essentially using rapid deferential rt-PCR test for molecular screening of both viruses in equine abortion cases as confirmed the contribution of both virus in the same sample EHV4 1 in addition there is an incidence of reactivation of the latent viruses .Moreover the sensitivity and specificity of viral nucleic acid detection using n-PCR and semi nested PCR agreed in many previous studies which improve the epidemiological investigation and circulation in horse population (Amer et al., 2011; Al-Shammari et al., 2016).

The virus propagation on the CAM of ECE was successful, especially in the samples with high C.T. in rt-PCR where eleven inoculation propagated and displayed a positive cytopathological change as a thickening and diffuse opacity known pock lesion, this result confirms the EHV member isolation as previously done by (Hassanien et al., 2002), and the results proved the higher correlation between the C.T. value in rt -PCR and the possibility of successful Isolation of EHV-4 due to higher viral load in the sample this in agreement with (Pavulraj et al., 2021) where virus isolated from samples with C.T. lower than 25 and the samples with late C.T. not isolated successfully.

The genomic DNA of EHV-4 was detected in horse fetal liver and spleen isolates by successful amplification of a specific 580 pb fragment of virus gB using the n-PCR second cycle, confirming the circulation of EHV-4 and playing a critical role in recurrent abortion outbreaks in Egyptian horses as reported in previous occasions in Egypt (Al-Shammari et al., 2016; Amer et al., 2011; Ghoniem et al., 2018), the finding also prove the superiority of n-nested PCR as a molecular test over the other serological assays. n-PCR could be used as a test of choice in EHV-4 diagnostic due to its accuracy and higher sensitivity up to one viral DNA copy, which overcomes the negativity of serological assays in recently or latently infected horses and reasonable cost with higher sensitivity, especially in low resources countries and where rt-PCR not available, many assays were developed and used by (Borchers and Slater, 1993; Varrasso et al., 2001; Wang et al., 2007).

The phylogenetic analysis with construction of maximum-likelihood tree for the gB sequences from isolates reveals its homology and identity up to 100 % of the three Egyptian isolates with EHV-4 isolates and strains available in GenBank from different localities worldwide as Japan, Australia, Germany, Turkey, China and Ireland, 99% identity with one isolate from Egypt, they were in the same clade with eleven sequences, The Analysis prove that all have the same gB and herpes virus have a low rate of nucleotide substitution and a very stable genomic DNA (Thiry et al., 2005), however we funded one SNP from G to C at position 247 in the isolate from China , another from G to A in isolates from Egypt and turkey at position 405 of our isolate's sequences , this difference could change on the pathogenicity and lead to alteration in the virus behavior and clinical presentation as occurs in EHV-1 where a point mutation in DNA polymerase was associate with neuropathogenic outbreaks (Nugent et al., 2006), so we recommend further deep investigation by whole genome sequencing for EHV-4 from abortion outbreaks to address any recombination occur .

The EHV-8 was close to EHV-4 in the BLSAT results where similarity revealed less than 87% identity at the nucleotides level and up to 8 amino acids differences in a different position and the same for EHV-4, this distance at genomic level explain the differentiation and large distance between the EHV-1 and -4 subtypes role of gB in cellular tropism, pathogenicity, and epidemiology as studied by (Spiesschaert et al., 2015; Vaz et al., 2016).

This study highlight that EHV-4 virus reactivation and or spread is possible to mares by direct and indirect routes agree with (Vargas-Bermudez et al., 2018), so the stressors play an essential role in the disease outbreaks. Measures are necessary to alleviate stressors in equine studs must be applied as the disease could occur at any time of the year (Matsumura et al., 1992). The result of phylogenetic analysis has shown that EHV4 were homogenous and grouped in one cluster as in the previous study from and out of Egypt, and this study present and confirm the role of EHV-4 as one of the major viral disease causing equine abortion in Egyptian mares, we recommend the strict application of biosecurity measures that will help in minimizing the occurrence of disease outbreaks, quarantine measures with restriction for any new horse entry to the farm, continuous virus monitoring, and regular surveillance and horses immunization. A more epidemiological and molecular investigation is required to address this alteration in the known viral pathogenicity and vaccines efficacy.

CONCLUSIONS AND RECOMMENDATIONS

In conclusion, EHV-4 is a probable causative agent for equine abortion, the virus infection in aborted fetuses from an outbreak in Egyptian mares was evidenced by rt-PCR, virus isolation, n-PCR, and viral gB sequencing; the disease could result from EHV-4 reactivation in latently infected equine or a true new infection both present a deviation in the previously known form of EHV-4 as a respiratory pathogen, and now it plays an important role in abortion requires more investigation.

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NOVELTY STATEMENT

The study confirms the considerable role of EHV-4 as a causative agent for equine abortion, and proves the circulation of EHV-4 during an outbreak of abortion ion 2019 in some Egyptian equine studs as the first report in last years, this study also emphasizes the alteration of the viral pathogenicity as a known respiratory pathogen cause rhinopneumonitis to abortive one, the rt-PCR reveal higher efficacy in viral detection, and we recommend it as a rapid routine diagnostic test. viral gB revealed a higher similarity among EHV-4 isolated from Egypt and globally.

AUTHOR'S CONTRIBUTION

Omnia Khattab, Naglaa M. Hagag, Mervat Hamdy, Mohamed Mashaly: Conceptualization and study design, samples collection, Conducted the experiments and Acquisition of data. data. Elsayyad Ahmed, Mervat E. Hamdy, Hala kamel, Omnia Khatab; Drafted the manuscript, carried out the analysis and data validation. Ayman Hamed, Essam I. Ibrahiem, Hanan Fahmy ,Momtaz A. Shahein and Elsayyad Ahmed; critical revision, data accuracy, supervision and and approval of the final version of the manuscript to be published

All authors read and approved the final manuscript.

Conflict of interest

The authors have declared no conflicts of interest.

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